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Flagellin and lipopolysaccharide up-regulation of IL-6 and CXCLi2 gene expression in chicken heterophils is mediated by ERK1/2-dependent activation of AP-1 and NF-κB signaling pathways

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The Toll-like receptor agonists, flagellin (FLG) and lipopolysaccharide (LPS), stimulate chicken heterophils to induce the expression and secretion of pro-inflammatory cytokines by a mechanism involving the triggering of differential MEK–ERK signaling cascades. However, the translocation and activation of transcription factors potentially involved in the control of cytokine gene expression remains unknown. Herein, we examined the effects of FLG and LPS on the activation of the transcription factors NF-κB and AP-1 and their role in regulating heterophil activation leading to cytokine gene expression. Treatment of heterophils with either FLG or LPS induced a significant increase in DNA binding by the NF-κB family members p50, c-Rel, and RelB. Likewise, FLG and LPS induced a significant increase in DNA binding by the AP-1 family members c-Jun and JunD. The activation of both NF-κB and AP-1 was inhibited following treatment of heterophils with specific inhibitors of ERK1/2 (U0126 and PD098059), NF-κB (Bay 11-7086 and the cell-permeable NF-κB peptide, SN50), and AP-1 (Tanshinone IIA). Likewise, the up-regulation of gene expression of the pro-inflammatory cytokine, IL-6, and the inflammatory chemokine, CXCLi2, were inhibited when heterophils were treated with the same specific inhibitors. Taken together these data demonstrate that FLG and LPS stimulate the up-regulation of expression of IL-6 and CXCLi2 through an ERK1/2-dependent activation of both NF-κB and AP-1.

Keywords: Flagellin, lipopolysaccharide, IL-6, CXCLi2, chicken heterophils, ERK1/2, AP-1 and NF-κB signaling pathways

INTRODUCTION

Polymorphonuclear leukocytes (PMNs) are vital cellular components of innate immunity and function by killing pathogenic microbes following phagocytosis. The primary PMN in poultry is the heterophil, the avian equivalent to the mammalian neutrophil. Like the neutrophil, heterophils provide a rapid deployment of the effector arm of the bird's innate immune system. They display a variety of pathogen recognition receptors (PRRs), including Toll-like receptors (TLRs), which account for the recognition of a multitude of invading microbes.[1–7] We have found that heterophils constitutively express eight of the ten known chicken TLRs which, when stimulated with specific TLR agonists, functionally activate heterophil oxidative burst and degranulation and induce the up-regulation of expression of pro-inflammatory cytokines (IL-1β, IL-6) and chemokines (CXCLi2, CCLi4).[8–11]

In mammals, the triggering of cells of the innate immune system (macrophages, granulocytes, and dendritic cells) through TLRs initiates a canonical signal transduction cascade that includes the downstream activation of the cytoplasmic portion of the TLR, the Toll/IL-1 receptor (TIR) domain. This results in the recruitment of MyD88/IRAK/TRAF6 that activates the

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mitogen-activated protein kinase (MAPK) superfamily cascade, culminating in the activation of the transcription factors nuclear factor-κB (NF-κB) and activator protein-1 (AP-1).[12–14] These transcription factors collaborate to induce transcription and expression of a large number of downstream genes that participate in the innate immune response, including pro-inflammatory cytokines and chemokines.

The signaling pathways of TLR have been comprehensively studied in mammals, but not in lower vertebrates, although the signaling proteins that are activated are also conserved across species.[15,16] In birds, we have been defining the signaling events following TLR activation of the heterophil. We have previously reported the activation of all three members of the MAPK family, extracellular-regulated kinase (ERK1/2), c-Jun N-terminal kinase (JNK), and p38, following flagellin (FLG) or LPS stimulation of avian heterophils.[5]

The current study was initiated to identify the transcription factor signaling pathways that, triggered downstream of ERK1/2 activation, induce pro-inflammatory cytokine and chemokine gene expression following heterophil stimulation by FLG and LPS.

Materials and Methods

Experimental chickens

Leghorn chickens (Hy-Line W-36) were obtained on the day-of-hatch from a commercial hatchery (Hy-Line International, Bryan, TX, USA) and placed in floor pens on pine shavings. Birds were provided water and a balanced, unmedicated ration *ad libitum*. The feed ration contained or exceeded the levels of critical nutrients recommended by the National Research Council.[18]

Reagents

Ultra-pure lipopolysaccharide (from *Salmonella enterica* sv. Minnesota; LPS) and flagellin (from *Salmonella enterica* sv. Typhimurium; FLG) were purchased from InVivoGen (San Diego, CA, USA) and prepared in sterile physiological water as per the manufacturer's instructions. The inhibitors U0126 and PD098059 (for inhibiting ERK 1/2) were purchased from Tocris Bioscience (Ellisville, MO, USA) and Calbiochem (La Jolla, CA, USA), respectively. Tannishone IIA (AP-1 inhibitor), the IκB phosphorylation inhibitor, BAY 11-7086, and SN50, a cell-permeable, inhibitory peptide of the nuclear translocation of NF-κB, were purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA, USA). Tannishone IIA, U0126, and PD098059 were dissolved in DMSO and stock solutions were stored at 4°C until used. Working concentrations of the inhibitors were prepared in RPMI 1640 tissue culture medium from the stock solutions. The final concentration of DMSO in the experiments was less than 0.5%.

Isolation of peripheral blood heterophils

Avian heterophils were isolated from the peripheral blood of day-old chickens as described previously.[5] Briefly, disodium ethylenediaminetetraacetic acid (EDTA)-anti-coagulated blood was mixed with 1% methylcellulose (25 centiposes; Sigma Chemical Co., St Louis, MO, USA) at a 1.5:1 ratio and centrifuged at 25 *g* for 30 min. The serum and buffy coat layers were retained and suspended in Ca^{2+},Mg^{2+}-free Hanks' balanced salt solution (HBSS, 1:1; Sigma Chemical Co.). This suspension was layered over a discontinuous Ficoll-Hypaque (Sigma Chemical Co.) gradient (specific gravity 1.077 over specific gravity 1.119). The gradient was then centrifuged at 250 *g* for 60 min. After centrifugation, the 1.077/1.119 interfaces and 1.119 band containing the heterophils were collected and washed twice in RPMI 1640 medium (Sigma Chemical Co.) and resuspended in fresh RPMI 1640. Cell viability was determined by Trypan Blue exclusion. The purity of the heterophil suspensions was assessed by microscopic examination of Hema-3 stained (Curtin Mathison Scientific, Dallas, TX, USA) cytospin (Shandon Scientific, Pittsburgh, PA, USA) smears. Heterophil preparations obtained by this method were typically > 98% pure and > 95% viable. On average, the other 2% was comprised of monocytes (at most 0.5%), lymphocytes (at most 0.8%), and thrombocytes (at most 0.7%). The cell concentration was adjusted to 1 x 10^7 heterophils/ml and stored on ice until used.

Inhibitor treatments

Heterophils isolated as described above were aliquoted into sterile 2-ml Eppendorf tubes (1 x 10^7 cells/ml) where they were pre-incubated with the appropriate concentrations of the various signal transduction inhibitors for 30 min at room temperature. Following these pre-incubations, the heterophils were stimulated with either FLG or LPS as described below. The following inhibitors and optimal concentrations were used in these studies: U0126 and PD098059 (ERK1/2 inhibitors; 50 μM); Tannishone IIA (AP-1 inhibitor; 10 μg/ml), BAY 11-7086 (IκB phosphorylation inhibitor; 50 μM) and SN50 (NF-κB inhibitor, 100 μg/ml) as previously determined.[1–3,9,11,17] Based on our previous experiments, the optimal concentrations used in the present experiments had no toxic effects on the avian heterophils.[1–3,9,11,17] In

Table 1. Real-time quantitative RT-PCR probes and primers

RNA target		Probe/primer sequence	Accession number[a]
28S	Probe	5′-(FAM)-AGGACCGCTACGGACCTCCACCA-(TAMRA)-3′	X59733
	Forward	5′-GGCGAAGCCAGAGGAAACT-3′	
	Reverse	5′-GACGACCGATTGCACGTC-3′	
IL-6	Probe	5′-(FAM)-AGGAGAAATGCCTGACGAAGCTCTCCA-(TAMRA)-3′	AJ250838
	Forward	5′-GCTCGCCGGCTTCGA-3′	
	Reverse	5′-GGTAGGTCTGAAAGGCGAACAG-3′	
CXCLi2	Probe	5′-(FAM)-TCTTTACCAGCGTCCTACCTTGCGACA -(TAMRA)-3′	AJ009800
	Forward	5′-GCCCTCCTCCTGGTTTCAG-3′	
	Reverse	5′-TGGCACCGCCAGCTCATT-3′	

[a]Genomic DNA sequence.

addition, the specificity of U0126 and PD098059 as specific inhibitors of ERK1/2 in avian immune cells (heterophils and monocytes) have been demonstrated previously.[11,17] The specificity of Tannishone IIA, BAY 11-7086, and SN50 were evaluated in these experiments.

TLR stimulation

TLR agonists were used at the optimal concentrations (FLG, 100 ng/ml; LPS, 10 μg/ml)) as previously described.[5] Heterophils were stimulated in 2-ml Eppendorf tubes and incubated at 41°C and 5% CO_2 for 1 h.

NF-κB and AP-1 activation analysis

The ELISA-based Trans-Am transcription factor kits (Active Motif, Carlsbad, CA, USA) were used to detect and quantify NF-κB and AP-1 activation. These kits use a patented technology to attach oligonucleotides containing either a TPA-responsive element (5′-TGAGTCA-3′) or an NF-κB binding consensus sequence (5′-GGGACTTTCC-3′) to a 96-well plate according to the transcription factors analyzed.[19,20] The active forms of the subunits for AP-1 (c-Fos, FosB, Fra-1, Fra-2, c-Jun, JunB, JunD) or NF-κB (p65, p52, p50, c-Rel, RelB) in whole cell extracts can be detected using specific antibodies for epitopes that are accessible only when the nuclear factors are activated and bound to their target DNA.

Preparation of cell extract was done according to the manufacturer's instructions. The specificity of the assays was checked by measuring the ability of soluble wild-type or mutated NF-κB or AP-1 oligonucleotides to inhibit binding. The results are expressed as specific binding (absorbance measured in the presence of the mutated oligonucleotides minus that measured in the presence of the wild-type oligonucleotides) according to the manufacturer's instructions.

Isolation of total RNA

Total RNA was prepared from heterophil preparations using the RNeasy mini kit (Qiagen) following the manufacturer's instructions, eluted with 50 ml RNase-free water, and stored at –80°C until qRT-PCR analyses were performed.

Quantitative real-time RT-PCR

Cytokine mRNA expression was quantitated using a previously described method.[9] Primers and probes for cytokines, chemokines and 28S RNA-specific amplification have been described but for clarity are provided (Table 1).[9] The qRT-PCR was performed using the TaqMan fast universal PCR master mix and one-step RT-PCR master mix reagents (Applied Biosystems, Cheshire, UK). Amplification and detection of specific products were performed using the Applied Biosystems 7500 Fast Real-Time PCR System with the following cycle profile: one cycle of 48°C for 30 min, one cycle of 95°C for 20 s, 40 cycles of 95°C for 3 s, 60°C for 30 s. Quantification was based on the increased fluorescence detected by the 7500 Fast Sequence Detection System due to hydrolysis of the target-specific probes by the 5′-nuclease activity of the *rTth* DNA polymerase during PCR amplification. The passive reference dye 6-carboxy-χ-rhodamine, which is not involved in amplification, was used for normalization of the reporter signal. Results are expressed in terms of the threshold cycle value (C_t), the cycle at which the change in the reporter dye passes a significance threshold (R_n).

To account for variation in sampling and RNA preparation, the C_t values for cytokine- or chemokine-specific product for each sample were standardised using the C_t value of 28S rRNA product for the same sample. To normalise RNA levels between samples within an experiment, the mean C_t value for 28S rRNA-specific product

was calculated by pooling values from all samples in that experiment. Tube-to-tube variations in 28S rRNA C_t values about the experimental mean was calculated. The slope of the 28S rRNA $\log_{10}$ dilution series regression line was used to calculate differences in input total RNA. Using the slopes of the respective cytokine, chemokine or 28S rRNA $\log_{10}$ dilution series regression lines, the difference in input total RNA, as represented by the 28S rRNA, was then used to adjust cytokine- and chemokine-specific C_t values, as follows:

$$\text{Corrected } C_t \text{ value} = C_t + (N_t - C_t')* S/S' \qquad \text{Eq. 1}$$

where C_t = mean sample C_t, N_t = experimental 28S mean, C_t' = mean 28S of sample, S = cytokine or chemokine slope, S′= 28S slope.

Statistical analysis

The anti-coagulated blood from 50 chickens was pooled and heterophils were isolated from each treatment group as described above. At least three replicates were conducted for each assay with the heterophils from each pool of chickens. The data from these four repeated experiments were pooled for presentation and statistical analysis. The mean and SEM were calculated for each of the treatment groups. Differences between the non-stimulated heterophils and the agonist-stimulated heterophils were determined by analysis of variance. Significant differences were further separated using Duncan's multiple range test. The data obtained using heterophils stimulated with each TLR agonist were compared to non-stimulated control heterophils.

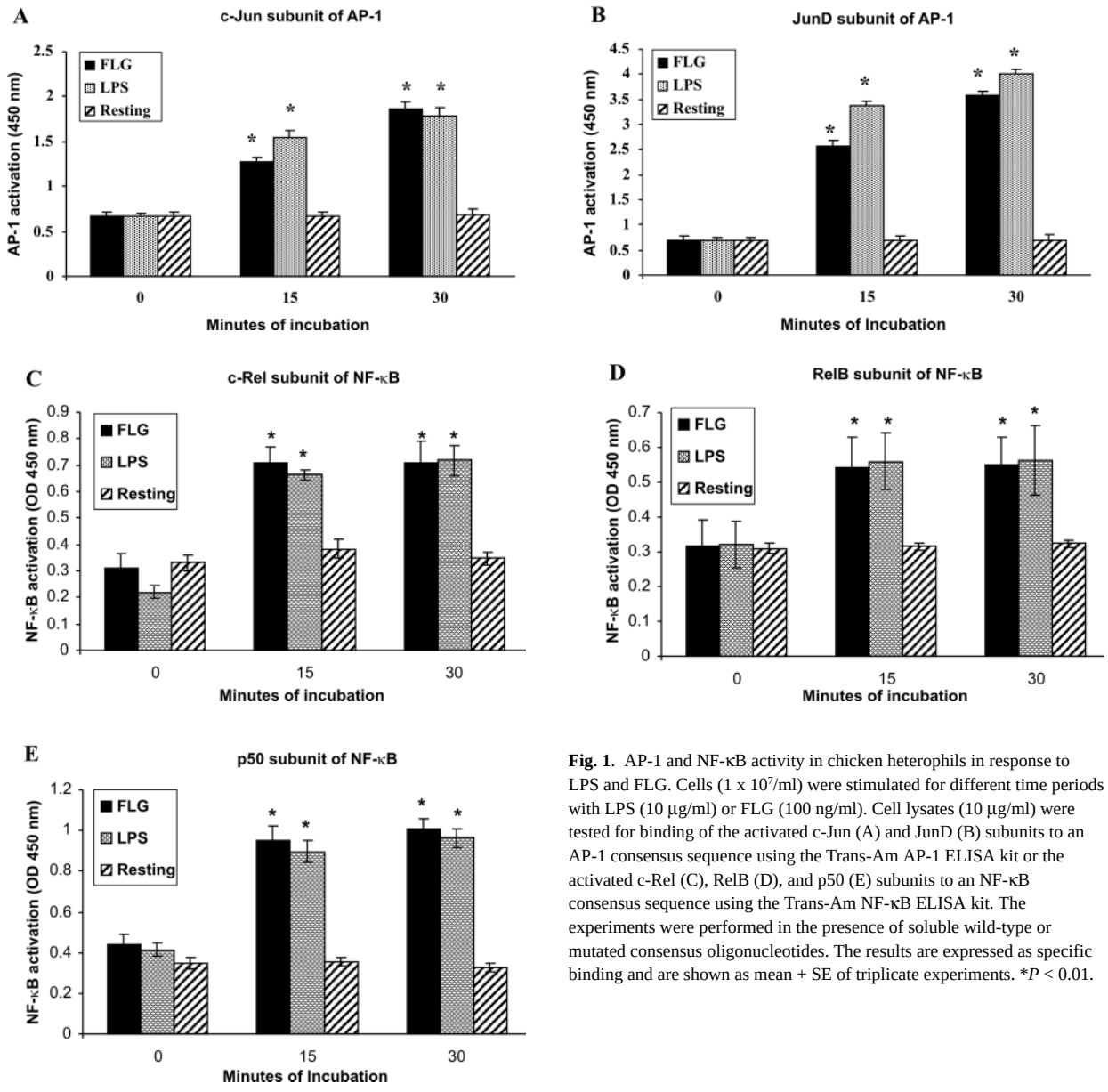

Fig. 1. AP-1 and NF-κB activity in chicken heterophils in response to LPS and FLG. Cells (1 x 10^7/ml) were stimulated for different time periods with LPS (10 µg/ml) or FLG (100 ng/ml). Cell lysates (10 µg/ml) were tested for binding of the activated c-Jun (A) and JunD (B) subunits to an AP-1 consensus sequence using the Trans-Am AP-1 ELISA kit or the activated c-Rel (C), RelB (D), and p50 (E) subunits to an NF-κB consensus sequence using the Trans-Am NF-κB ELISA kit. The experiments were performed in the presence of soluble wild-type or mutated consensus oligonucleotides. The results are expressed as specific binding and are shown as mean + SE of triplicate experiments. *$P < 0.01$.

RESULTS

Activation of AP-1 and NF-κB by LPS and FLG

Treatment of the heterophils with either FLG or LPS induced a significant increase in DNA binding by the AP-1 family members, c-Jun and JunD (Fig. 1A,B). No other increase in DNA binding by the other AP-1 family members (c-Fos, FosB, Fra-1, Fra-2, or JunB; data not shown) was observed. Likewise, FLG and LPS induced a significant increase in DNA binding by the NF-κB family members, c-Rel, RelB, and p50 (Fig. 1C–E). We detected a significant increase in AP-1 and NF-κB activation within 15 min of stimulation of heterophils with FLG and LPS when compared to non-stimulated control heterophils. However, it should be pointed out that Western blot analysis of the chicken heterophils and mouse macrophages using the anti-p65 and p52 antibodies from the TransAm kit showed no cross-reactivity with chicken p65 or p52, respectively (data not shown). Therefore, we cannot rule out the activation of either the p65 or p52 subunit during chicken heterophil activation. According to the manufacturer, the antibody against p65 was mapped to the N-terminal region of the gene where there is less than 30% homology between the chicken p65 and murine p65, whereas, the antibody against p58 was mapped to an area of the gene where there is less than 25% homology between the chicken p52 and murine p52.

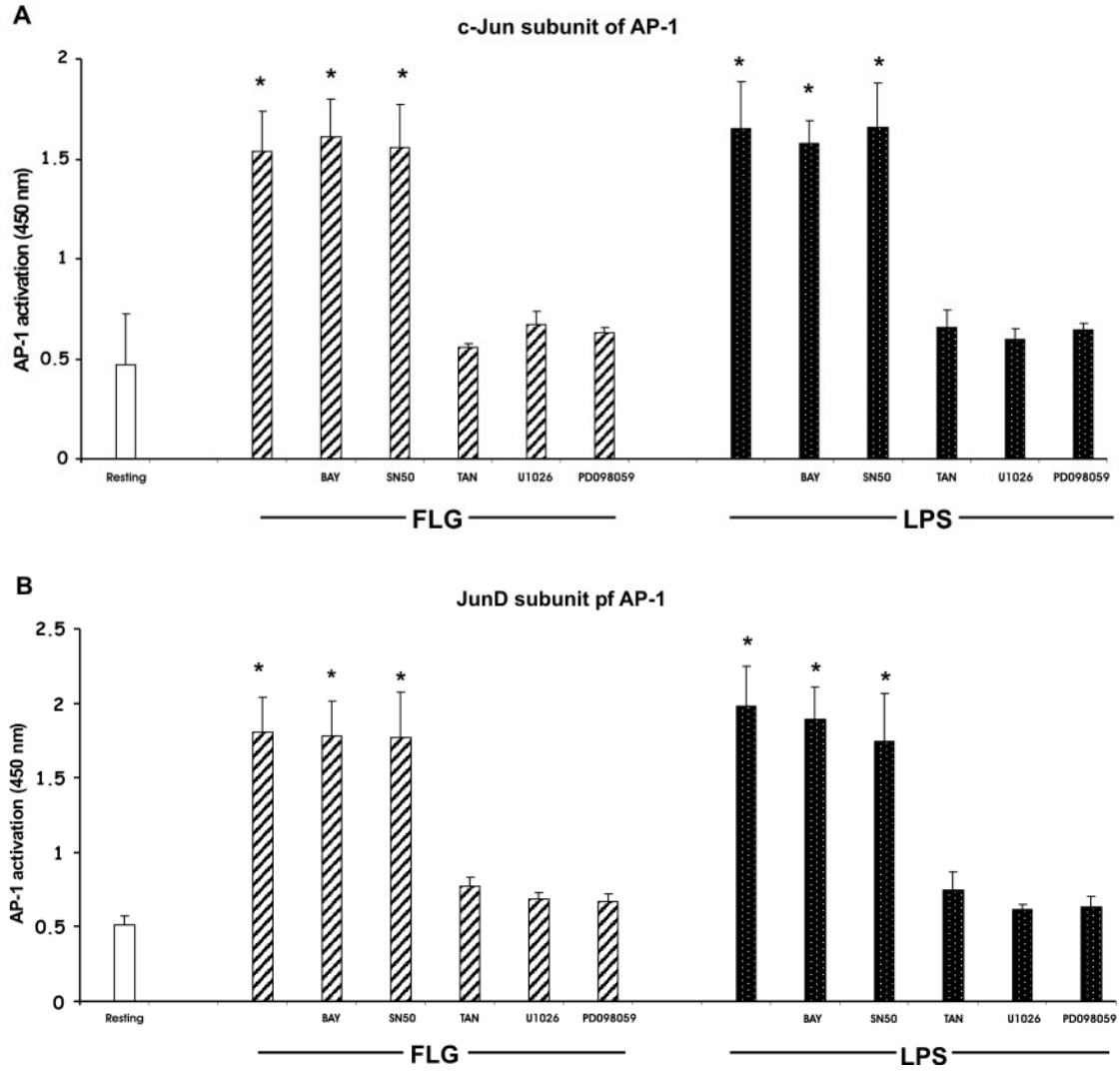

Fig. 2. Effect of ERK 1/2, AP-1, or NF-κB inhibitors on binding of either c-Jun and JunD subunits of AP-1 to an AP-1 binding consensus sequence or c-Rel, RelB, and p50 subunits to an NF-κB consensus sequence. Heterophils were stimulated with FLG or LPS in the presence or absence of either the ERK1/2 inhibitors (U0126, 50 μM; PD098059, 50 μM), AP-1 inhibitor (Tannishone IIA (10 μg/ml) or the NF-κB inhibitors (Bay 11-7086, 50 μM ; SN50, 100 μg/ml). Cell lysates (10 μg/ml) were tested for binding of the activated c-Jun (A) and JunD (B) subunits to an AP-1 consensus sequence using the Trans-Am AP-1 ELISA kit or the activated c-Rel (C), RelB (D), and p50 (E) subunits to an NF-κB consensus sequence using the Trans-Am NF-κB ELISA kit. The experiment was performed in the presence of soluble wild-type or mutated consensus oligonucleotides. The results are expressed as specific binding and are shown as mean + SE of triplicate experiments $^{**}P < 0.01$; $^{*}P < 0.05$. (continued on next page)

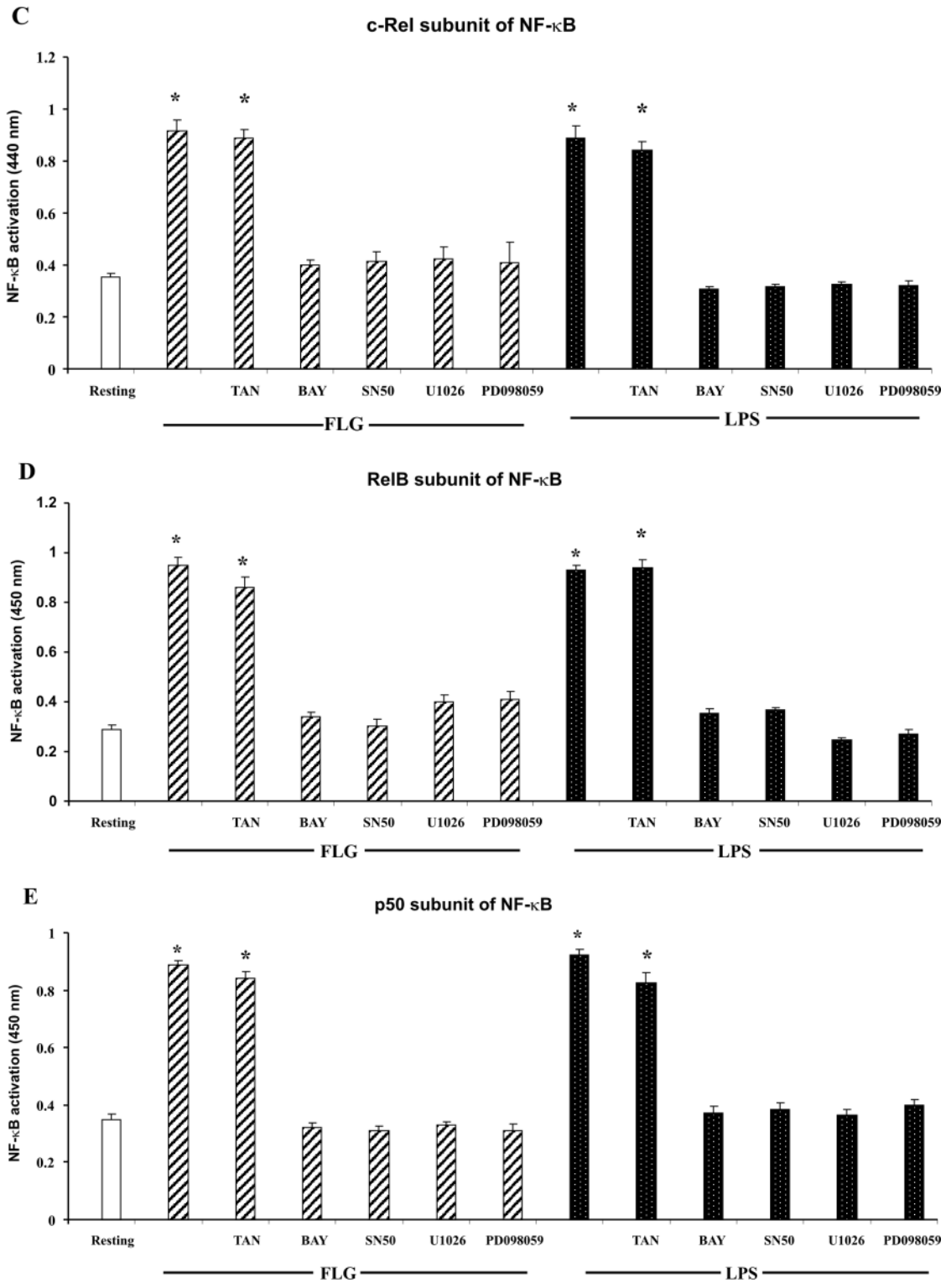

Fig. 2. (*Continued*) Effect of ERK 1/2, AP-1, or NF-κB inhibitors on binding of either c-Jun and JunD subunits of AP-1 to an AP-1 binding consensus sequence or c-Rel, RelB, and p50 subunits to an NF-κB consensus sequence. Heterophils were stimulated with FLG or LPS in the presence or absence of either the ERK1/2 inhibitors (U0126, 50 μM; PD098059, 50 μM), AP-1 inhibitor (Tannishone IIA (10 μg/ml) or the NF-κB inhibitors (Bay 11-7086, 50 μM ; SN50, 100 μg/ml). Cell lysates (10 μg/ml) were tested for binding of the activated c-Jun (A) and JunD (B) subunits to an AP-1 consensus sequence using the Trans-Am AP-1 ELISA kit or the activated c-Rel (C), RelB (D), and p50 (E) subunits to an NF-κB consensus sequence using the Trans-Am NF-κB ELISA kit. The experiment was performed in the presence of soluble wild-type or mutated consensus oligonucleotides. The results are expressed as specific binding and are shown as mean + SE of triplicate experiments $^{**}P < 0.01$; $^{*}P < 0.05$.

ERK1/2 regulates activation of transcription factors

As expected, activation of the transcription factors AP-1 (Fig. 2A,B) and NF-κB (Fig. 2C–E) was totally blocked by pretreatment of the cells with selective inhibitors of either AP-1 (Tannishone IIA; Fig. 2A,B) or NF-κB (Bay 11-7086 and SN50; Fig. 2C–E). However, pretreatment of the heterophils with the selective ERK1/2 inhibitors (U1026 or PD098059) also totally blocked the activation of both AP-1 (Fig. 2A,B) and NF-κB (Fig. 2C–E). These data provide direct evidence of an ERK1/2-dependent regulation of transcription factors in chicken heterophils stimulated with FLG or LPS.

ERK1/2 activation is a key event in IL-6 and CXCLi2 gene expression

We next examined the effect of the ERK1/2 inhibitors, U0126 and PD098059, on the expression of IL-6 and CXCLi2 in avian heterophils. The FLG- and LPS-induced expression of IL-6 (Fig. 3A) and CXCLi2 (Fig. 3B) was blocked by pretreatment of the cells with both U0126 and PD098059.

As expected, treatment with either the selective AP-1 inhibitor, Tannishone IIA, or the selective NF-κB inhibitors, Bay 11-7086 and SN50, blocked the FLG- and LPS-induced expression of IL-6 (Fig. 3A) and CXCLi2 (Fig. 3B) in avian heterophils.

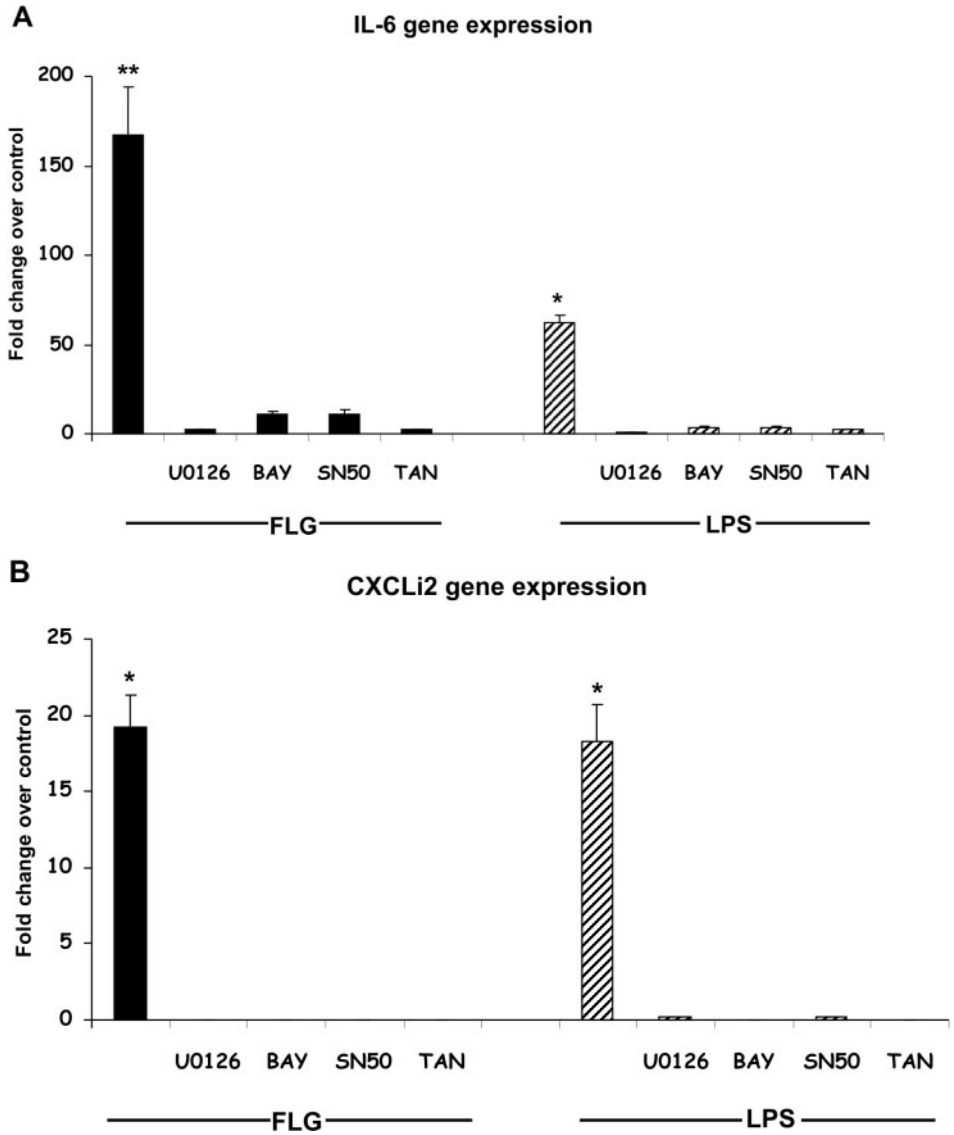

Fig. 3. Effect of ERK 1/2, AP-1, and NF-κB inhibitors on IL-6 (A) and CXCLi2 (B) gene expression in chicken heterophils. Cells were cultured in the presence or absence of highly selective inhibitors (U0126, 50 μM; PD098159, 50 μM; Bay 11-7086, 50 μM; SN50, 100 μg/ml; or Tannishone IIA (10 μg/ml) for 1 h and stimulated for 1 h with either LPS or FLG. Total RNA was extracted and quantitated using TaqMan technology. Data are expressed as the fold-change in cytokine mRNA levels when treated samples were compared to non-stimulated control heterophils from age-matched chickens. Error bars show SEM from triplicate samples from three separate qRT-PCR experiments. $P < 0.01$.

DISCUSSION

As the first cell type encountering and interacting with Gram-negative and Gram-positive bacterial pathogens, heterophil recognition of potential pathogenic microbes by PRRs, especially the TLRs, is vital for the health of the bird. We have found that heterophils constitutively express eight of the known chicken TLRs.[5] Recognition of PAMPs by TLRs leads to the activation of intracellular signaling pathways that results in the production of defensins (Kaiser and Kogut, unpublished results) and pro-inflammatory mediators including cytokines, chemokines, and arachidonic acid metabolites.[5,8,9] However, our understanding of the signaling events that control the gene expression and production of these inflammatory mediators in avian heterophils is incomplete. We have previously shown that FLG and LPS from *S. enterica* stimulate pro-inflammatory cytokine and chemokine gene expression via ERK1/2 kinase cascade in the heterophil.[5,17] However, the importance of the phosphorylation of ERK1/2 kinase in these signaling pathways on transcription factor activation is unknown.

The present study revealed that FLG- and LPS-induced IL-6 and CXCLi2 gene expression was dependent upon early activation of AP-1 and NF-κB. We detected a significant increase in AP-1 and NF-κB activation within 15 min of stimulation of heterophils with FLG and LPS and a significant increase in IL-6 and CXCLi2 transcription in heterophils 1 h after stimulation with FLG or LPS. These sequential processes imply that AP-1 and NF-κB mediate the induction of IL-6 and CXCLi2 transcripts. This hypothesis is supported by the fact that we have previously identified potential AP-1 and NF-κB binding sites in the CXCLi2 promoter (one of each)[21] and in the IL-6 promoter (two AP-1 sites, one NF-κB).[22] Moreover, FLG- and LPS-induced expression of IL-6 and CXCLi2 was completely blocked by selective inhibitors of ERK1/2, AP-1, or NF-κB, which suggests that these pathways are likely involved in the up-regulation of these responses. Similar timing of transcription factor activation and cytokine gene expression was found in mammalian mononuclear and granulocytic cells stimulated with porins from *S. enterica* sv. Typhimurium cell membranes.[23,24] These findings further suggest that the activation of AP-1 and NF-κB and the phosphorylation of ERK1/2 are necessary prerequisites for increased pro-inflammatory cytokine and inflammatory chemokine expression in avian heterophils in response to *Salmonella* infection.[1–5,25,26]

The AP-1 transcriptional family consists of different combinations of Fos and Jun proteins. Members of the Jun family proteins (c-Jun, JunB, JunD) can homo-or heterodimerize among themselves or dimerize with Fos family proteins.[27,28] However, Fos family proteins (c-Fos, FosB, Fra-1, Fra-2) only dimerize with Jun proteins. In the present study, we found that FLG or LPS stimulation of heterophils activated AP-1, composed of Jun/Jun homodimers (c-Jun, JunD). No Fos proteins were activated in our system. Although Jun/Fos heterodimers have a higher DNA binding affinity than Jun/Jun homodimers,[27,28] the AP-1 activity measured in our experiments was sufficient to induce the expression of IL-6 and CXCLi2. This differs from human neutrophils, where cytokine expression was found to be independent of AP-1 activation.[29] However, Cloutier and colleagues[29] only examined the role of the JNK-dependent AP-1 signaling cascade in the induction of cytokine gene expression in human neutrophils. Conversely, members of the AP-1 transcription factor family are well known targets of the ERK1/2 pathway in a variety of cells, as was found in the present studies.[23,24,30–32]

Transcription factors of the NF-κB family remain in a quiescent state, complexed with inhibitory IκB proteins, in the cytosol of virtually all vertebrate cells. Upon activation, IκB proteins are phosphorylated and released from NF-κB, which then undergoes nuclear translocation and initiates gene transcription.[33] NF-κB is composed of homo- and heterodimer complexes made from the five subunits of the NF-κB family (p50, p65, p52, c-Rel, and RelB).[33,34] The phosphorylation of IκBα following cell activation induces the release of NF-κB dimers which translocate to the nucleus. In our assays, we found that FLG or LPS stimulation of heterophils activated NF-κB, composed of the p50, c-Rel, and/or RelB subunits. However, we cannot rule out the activation of either the p65 or p52 subunit during chicken heterophil activation due to unavailability of proper reagents to analyze the role of p65 and p52. Furthermore, treating the heterophils with either the IκB phosphorylation inhibitor, BAY 11-7086, or the cell-permeable, inhibitory peptide of the nuclear translocation of NF-κB, SN50, prevented the activation of NF-κB with a resulting decreased induction of IL-6 and CXCLi2 mRNA. These results are similar to those observed with human neutrophils, demonstrating the significant role of NF-κB activation in inducing the expression of pro-inflammatory cytokines and chemokines following stimulation with physiologically relevant stimuli such as TLR agonists and cytokines.[35]

In addition to the dependence of inducible pro-inflammatory cytokine and chemokine expression on AP-1 and NF-κB, we have also demonstrated that gene expression was regulated by the ERK1/2 MAPK pathway. Blockage of the ERK1/2 pathway by U0126 or PD098059 eliminated the expression of IL-6 and CXCLi2 in heterophils stimulated with FLG or LPS. Our results mirror those for human neutrophils, in that inhibitors of the MEK pathway significantly reduced the expression and release of all cytokines and chemokines examined.[24,35–39] The mechanism of ERK1/2 inhibition of inducible cytokine

and chemokine expression appears to be transcriptional, since the ERK blocker also prevented activation of both AP-1 and NF-κB (Fig. 2), suggesting a cross-talk between the ERK1/2 pathway and AP-1 and NF-κB. Interestingly, no such cross-talk was demonstrated between the MAPK pathway and these transcription factors in human neutrophils.[38] At this time, it is not known whether this cross-talk is unique to avian heterophils, but we have not found any reports in the literature that suggest such interactions in mammalian neutrophils. Further experiments are planned to compare cross-talk capabilities between MAPK pathways and transcription factors in avian and mammalian granulocytic cells.

CONCLUSIONS

We have shown that FLG and LPS transcriptionally induce IL-6 and CXCLi2 mRNA expression with the concurrent involvement of the transcription factors JunD, c-Fos, p50, c-Rel, and RelB. This is the first demonstration that the inducible expression of pro-inflammatory cytokines and chemokines in avian heterophils by TLR agonists is dependent upon the ability to phosphorylate selected MAPK pathways and activate AP-1 and NF-κB.

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